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THIAGO HENRIQUE ALMEIDA SOUZA

Pharmacological validation of the free-exploratory paradigm in Wistar rats: A proposed test of trait anxiety

São Cristóvão

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Trabalho de Conclusão de Curso apresentado ao Departamento de Farmácia da Universidade Federal de Sergipe como requisito parcial para obtenção do Bacharelado em Farmácia.

Orientadora: Profª. Dra. Flavia Teixeira Silva

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Aprovada em: ____/____/____

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PARECER

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Dedico este trabalho primeiramente a Deus, por ser essencial em minha vida, autor de meu destino, o meu guia. Aos meus pais, Aída e Gilrobson, que são responsáveis por alicerçar o meu caminho, por serem minha fortaleza. À minha avó Helena (In memorian), pelo amor, carinho e respeito que sempre fizeram eu me sentir uma pessoa especial. À minha professora orientadora, Dra. Flavia Teixeira, pelo auxílio e pelos ensinamentos. E, em especial, ao grande Mestre Tiago Costa, pela paciência, dedicação e confiança inenarráveis.

ARTIGO

Pharmacological validation of the free-exploratory paradigm in Wistar rats: A proposed test of trait anxiety

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Abstract

The free-exploratory paradigm (FEP) has been proposed as a model of trait anxiety for both mice and rats. However, its pharmacological validation has only been carried out for mice. Thus, the aim of the present study was to pharmacologically validate FEP for Wistar rats, by testing the effects of clinically established anxiolytic and anxiogenic drugs, in four different experiments. In all experiments, male Wistar rats were first tested in FEP to be categorized according to their levels of trait anxiety (high, medium and low). Then, only medium trait anxiety rats were selected to be tested again in FEP, two weeks later, after being pharmacologically treated, according to each experiment as follows: Experiment I: 0.5 mg/kg of diazepam (DZP) or vehicle; Experiment II: 20 mg/kg of pentylenetetrazole (PTZ) or vehicle; Experiment III: 5 mg/kg of fluoxetine (FLX5) or vehicle: and Experiment IV: 0.5 mg/kg of fluoxetine (FLX0.5) or vehicle. As a group, the results showed that PTZ and FLX5 increased levels of trait anxiety and reduced locomotor activity, whereas DZP and FLX0.5 decreased levels of trait anxiety, without impairing locomotor activity. These results demonstrate that FEP for rats is able to predict clinical anxiolytic and anxiogenic activities of different drugs, including fluoxetine, which is believed to present a dual effect on anxiety. Therefore, this paradigm can be proposed as an effective method for testing potential trait anxiety-reducing drugs, in rats.

1. Introduction

Anxiety disorders are associated with significant impairment in quality of life and negative interferences on occupational, academic and social contexts (Olatunji et al., 2007), being the most prevalent class of psychiatric disorders in the general population (Kessler et al., 2005) and the third most expensive brain disorder in Europe, with an economic cost of billions of euros (Olesen et al., 2012). However, its treatment is still challenging, as the drugs used for the relief of anxiety symptoms can have important side-effects, promote therapeutic dependence, or present a delay in their onset of action (Starcevic, 2005). Furthermore, not all patients benefit from the available treatments, and only a few of them have a response near to complete recovery (Ravindran, 2010). These facts justify the growing number of studies in order to develop more effective drugs, with fewer side-effects, for the control of anxiety disorders.

For the experimental evaluation of new drugs with a potential anxiolytic effect, the scientific community counts on various animal models. However, most of these models confront the animals with an anxiety provoking situation, either through anxiogenic chemicals (β-carbolines, yohimbine, caffeine), conflict tests (Geller and Seifter box, light/dark chamber, elevated plus-maze) or exposure to aversive stimuli (defensive burying; Martin, 1998; Garner et al., 2009; Treit, 2010; McGonigle, 2014), thus modelling state anxiety, which is the anxiety a subject experiences at a particular moment in time, in response to a threatening situation. However, in the study of anxiety there is another important concept: trait anxiety, which describes individual differences, related to a tendency to present state anxiety, is relatively stable overtime (Spielberger,

1970; Treit, 2010) and is high in anxiety disorder patients (Bieling et al., 1998; Kennedy et al., 2001).

The underlying biological mechanisms of state and trait anxiety may not be the same (Treit et al., 2010). It has been shown that the anxiety response to a threatening stimulus involves brain structures such as amygdala, bed nucleus of the stria terminalis, septo-hippocampal system, median raphe nucleus, ventral periaqueductal grey matter and locus coeruleus (Gray and Mcnaughton, 2000; Brandão et al., 2003; Davis, 2006); while the anxious trait is thought to be related to the orbitofrontal cortex (Kalin et al., 2007; Blackmon et al., 2011). Therefore, it is reasonable to believe that a drug that is effective in animal models of state anxiety, and which may therefore reduce state anxiety in humans in threatening situations, might not be effective in reducing long-term anxiety in chronically anxious patients.

Taking all this into consideration, it becomes clear that pre-clinical tests for the development of new anxiolytic drugs should include animal models of trait anxiety.

To the best of our knowledge, the only test that has been proposed as an animal model o trait anxiety is the free-exploratory paradigm (FEP) (Griebel 1993, Teixeira-Silva et al., 2009; Oliveira et al., 2014). In this model, animals are given the opportunity to move around freely within an environment containing both familiar and novel parts. This approach allows the evaluation of neophobic responses. As the animals have a choice between novelty and familiarity, it is expected that individuals with low trait anxiety would exhibit a

preference for novelty, whereas high trait anxiety subjects would prefer familiarity.

FEP has been performed in both mice (Misslin et al. 1982; Misslin and Cigrang, 1986; Griebel et al., 1993; Belzung and Le Pape, 1994) and rats (Hughes, 1968; Matos et al., 2011; Goes et al., 2013), yet its pharmacological validation has only been carried out for mice (Belzung and Berton, 1997; Belzung et al., 2001).

With this in mind, the aim of the present study was to validate pharmacologically FEP in Wistar rats.

2. Material and methods

2.1. Animals

One hundred thirty-four adult (2–3 months) male Wistar rats were obtained from our own colony. The animals were kept five per cage (41 × 34 × 18 cm), in a temperature (22–24 °C) and light (12 h/12 h light/dark cycle; lights on at 06:00 a.m.) controlled room, with water and food *ad libitum*.

All procedures were approved by the local ethical committee (Universidade Federal de Sergipe) and complied with both national (Brazilian National Council on the Control of Animal Experimentation – Law 11.794, of October 8, 2008) and international guidelines (Council Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010) for the care of animals.

2.2. Free-exploratory paradigm

The Free-exploratory paradigm was set up as described by Antunes et al. (2011). The apparatus (Fig. 1) consisted of a wooden box, divided into two compartments, with each of these further subdivided into three exploratory units (20 x 20 cm), interconnected by small openings. The two compartments were separated by a removable partition. The box was placed on a stand in the rat home room. Approximately 24h before testing, the partition was installed and an animal was put into one half of the apparatus and left there until the test time, in order to become familiarized with it. This familiar half had fresh zeolites (Zoocel Biotério® - Celta Brasil, Cotia, BR) covering the floor and the animal had free access to food and water. On the test day the partition, between the familiar and the novel compartments was removed and the animal was observed for 15 minutes, under infra-red light. During this period, the following parameters were measured: total distance travelled (TDT), and the time spent in each compartment, from which the percentage of time spent in the novel side (%TNS) – a parameter considered a reliable measure of trait anxiety in rats (Teixeira-Silva et al., 2009). These parameters were measured using a computerized system for animal tracking (Anymaze, Stoelting Co., Wood Dale, Illinois, USA).

2.3. Drugs

Fluoxetine Hydrochloride (EMS – Sigma Pharma Ltda., Brazil), and pentylenetetrazole (Sigma-Aldrich™, USA) were dissolved in saline and

diazepam (EMS S/A, Brazil) was dissolved in 5% alcohol solution. All drugs were administered intraperitonially (i.p.), in a volume of 2 ml/kg body weight.

2.4. Procedure

Four experiments, following the same procedure, were performed aiming to pharmacologically validate FEP, in Wistar rats.

A total of 134 rats were first tested on the FEP (FEP1) as described above. The obtained results were used to classify them according to the %TNS, as presenting high (%TNS < 51.17), medium (51.17 $\leq$ %TNS $\leq$ 80.00) or low (%TNS > 80.00) levels of trait anxiety, in conformity with a previous categorization performed in our lab (Goes et al., 2013). After FEP1, only the animals with medium anxiety (n = 66) were selected for the study. They were put back into their home cages, and kept under standard conditions (described in the “Animals” section), until tested again in FEP (FEP2), two weeks later. In this second exposition, before the behavioural evaluation, the animals were pharmacologically treated as follows:

- Experiment I: 0.5 mg/kg of diazepam (DZP; n = 10) or vehicle (5% alcohol solution + saline, CTRL; n = 10), 30 minutes before evaluation.
- Experiment II: 20 mg/kg of pentylenetetrazole (PTZ; n=6) or vehicle (saline, CTRL; n=6), 15 minutes before evaluation.
- Experiment III: 5 mg/kg of fluoxetine (FLX5; n=8) or vehicle (saline, CTRL; n= 8), 30 minutes before evaluation.

- Experiment IV: 0.5 mg/kg of fluoxetine (FLX0.5; n=10) or vehicle (saline, CTRL; n=10), 30 minutes before evaluation.

Between each use, the free-exploratory boxes were emptied and then cleaned using a 10% ethanol solution.

All tests were performed in the dark phase of the light/dark cycle, between 6:00 and 7:00 p.m.

2.5. Statistical Analysis

The obtained data from all experiments were first analysed using Kolmogorov–Smirnov's test for normal distribution and Levene's test for the homogeneity of variances. No impediments to the use of parametric tests were found for any of the evaluated parameters.

The data were analysed by two-way ANOVA for repeated measures (factor 1: treatment; factor 2: trial). When the interaction between factors was significant, the analyses were followed fixing factor 1 and conducting one-way ANOVA for repeated measures; and fixing factor 2 and conducting one-way ANOVA for independent samples.

All significance tests were performed at the 5% significance level.

In all experiments, a z-score was calculated for all parameters and animals presenting values outside the between mean ± two standard deviations were excluded from the analysis.

3. Results

3.1. Experiment I (Fig. 2)

After the z-score calculation, the sample sizes for each group were as follows: CTRL (n=9) and DZP (n=9).

There was not significant interaction between treatment and trial for the TDT parameter ($F_{1,\ 16}$=1.318; p=0.267); therefore, the two main effects were analysed. Neither treatment [F(1,16)=3.4560; p=0.08] or trial [F(1,16)=0.657; p=0.429] changed TDT significantly.

Analysis of %TNS showed significant interaction between treatment and trial [F(1,16)=12.916; p=0.002]. Fixing factor treatment, analysis of the trial as a single factor showed that DZP spent more time in the novel side on FEP2 in comparison to FEP1 [F(1,8)=22.528; p=0.001], while no differences were found for CTRL [F(1,8)=3.855; p=0.08]. Fixing factor trial, analysis of the treatment as a single factor showed that DZP spent more time in the novel side in relation to CTRL on FEP2 [F(1,16)=7.696; p=0.013], while no differences were found on FEP1 [F(1,16)=2.421; p=0.139].

3.4 Experiment II (Fig. 3)

After the z-score calculation, the sample sizes for each group remained the same: CTRL (n=6) and PTZ (n=6).

There was significant interaction between treatment and trial for the TDT parameter [F(1,10)=18.979; p=0.001]. Fixing factor treatment, analysis of the trial as a single factor showed that the PTZ reduced TDT on FEP2 in relation to FEP1 [F(1,5)=44.558; p=0.001], but no differences were found for CTRL

[F(1,10)=0.292; p=0.612]. Fixing factor trial, analysis of the treatment as a single factor showed that PTZ reduced TDT in relation to CTRL on FEP2 [F(1,10)=11.933; p=0.006], but no differences were found on FEP1 [F(1,10)=0.076; p=0.789].

Analysis of %TNS showed significant interaction between treatment and trial [F(1,10)=10.135; p=0.009]. Fixing factor treatment, analysis of the trial as a single factor showed that PTZ spent less time in the novel side on FEP2 in comparison to FEP1 [F(1,5)=12.889; p=0.015], while no differences were found for CTRL [F(1,5)=0.251; p=0.637]. Fixing factor trial, analysis of the treatment as a single factor showed that PTZ spent less time in the novel side in relation to CTRL on FEP2 [F(1,10)=13.295; p=0.004], while no differences were found on FEP1 [F(1,10)=0.586; p=0.462].

3.2 Experiment III (Fig. 4)

After the z-score calculation, the sample sizes for each group remained the same: CTRL (n=8) and FLX5 (n=8).

There was a significant interaction between treatment and trial for the TDT parameter [F(1,13)=16.607; p=0.001]. Fixing factor treatment, analysis of the trial as a single factor showed that FLX5 reduced TDT on FEP2 in relation to FEP1 [F(1,7)=27.665; p=0.001], but no differences were found for CTRL [F(1,6)=0.152; p=0.710]. Fixing factor trial, analysis of the treatment as a single factor showed that FLX5 reduced TDT in relation to CTRL on FEP2 [F(1,14)=10.257; p=0.006], but no differences were found on FEP1 [F(1,14)=0.271; p=0.610].

Analysis of %TNS showed significant interaction between treatment and trial [F(1,13)=5.814; p=0.031]. Fixing factor treatment, analysis of the trial as a single factor did not reveal significant differences for either FLX5 [F(1,13)=5.042; p=0.059] or CTRL [F(1,6)=1.340; p=0.291]. Fixing factor trial, analysis of the treatment as a single factor showed that FLX5spent less time in the novel side in relation to CTRL on FEP2 [F(1,14)=5.298; p=0.037], while no differences were found on FEP1 [F(1,14)=0.650; p=0.433].

3.3 Experiment IV (Fig. 5)

After the z-score calculation, the sample sizes for each group were as follows: CTRL (n=9) and FLX0.5 (n=10).

There was no significant interaction between treatment and trial for the TDT parameter [F(1,17)=0.0425; p=0.839]; therefore, the two main effects were analysed. Neither treatment [F(1,17)=1.717; p=0.207)] or trial [F(1,17)=0.0004; p=0.985] changed TDT significantly.

Analysis of %TNS showed significant interaction between treatment and trial [F(1,17)=4.642; p=0.045]. Fixing factor treatment, analysis of the trial as a single factor showed that FLX0.5 spent more time in the novel side on FEP2 in comparison to FEP1 [F(1,179)=14.848; p=0.003], while no differences were found for CTRL [F(1,8)=0.480; p=0.507]. Fixing factor trial, analysis of the treatment as a single factor showed that FLX0.5 spent more time in the novel side in relation to CTRL on FEP2 [F(1,17)=6.278; p=0.022], while no differences were found on FEP1 [F(1,17)=0.240; p=0.630].

4. Discussion

The aim of the present study was to pharmacologically validate FEP in Wistar rats. In the pharmacological validation of FEP in mice (Griebel et al., 1993), two strains were used, BALB/c and C57BL/6, known respectively as “emotional” and “non-emotional”. As a result, anxiolytic effects were only observed in BALB/c mice, since the basal levels of novel side exploration of C57BL/6 mice was too high. Based on this, for the present study, only animals presenting medium trait anxiety were selected for the pharmacological tests, because their anxiety levels could either go up or down. This way, it was possible to observe both anxiolytic and anxiogenic effects in the same rat strain.

Three different drugs were used: 1) diazepam, which belongs to the class of benzodiazepines, which are typically used for the pharmacological validation of animal models (Andreatini, 2001); 2) pentylenetetrazole, a convulsant compound GABA-benzodiazepine receptor blocker (Huang et al., 2001), which induces anxiety in lower doses (subconvulsant; Pellow et al., 1985); and 3) fluoxetine, a selective serotonin reuptake inhibitor, which has been highlighted as the most prescribed drug for the relief of anxiety symptoms (Marshall, 2009).

Before initiating Experiment I, a pilot study was conducted in our laboratory using two doses of diazepam, 0.5 mg/kg and 2 mg/kg. According to the FDA (Center for Drug Evaluation and Research, 2002), these doses are equivalent to the ones that present anxiolytic effects in mice tested in FEP (1 mg/kg and 4 mg/kg, respectively; Griebel et al., 1993). However, the animals that received 2 mg/kg of diazepam remained motionless for almost the whole

test, preventing behavioural evaluation. Thus, 0.5 mg/kg was chosen to be tested. The results obtained from Experiment I showed that diazepam reduces trait anxiety, without impairing locomotor activity, in rats

Pentylenetetrazole (PTZ) used in Experiment II, increased levels of trait anxiety, as expected, and reduced locomotor activity. This hypolocomotor effect of PTZ is well known (Pellow, et al., 1985; Ramos et al., 1997; Ramos et al., 2008).

Similar results were observed with the administration of 5 mg/kg of fluoxetine (Experiment III). A locomotor activity reduction by fluoxetine has been observed before (Silva et al., 1999; Robert et al., 2011; Birket et al 2011) as well as its anxiogenic effect (Drapier et al., 2007; Birket et al 2011). Despite its extensive clinical use for the treatment of anxiety, fluoxetine displays controversial results in studies with animal models. Administered either acutely or chronically, fluoxetine can present anxiogenic, anxiolytic or no effects (De Vry et al., 2004; Borelli et al., 2004; Robert et al., 2011). Some differences in experimental protocols can be related to these conflicting results. For example, Silva and Brandão (2000) observed that chronic fluoxetine added to the drinking water had no effect in anxiety, while Robert et al. (2011) observed that chronic fluoxetine administered intraperitoneally had an anxiogenic effect. This result was observed in Wistar rats, receiving 5 mg/kg of fluoxetine; but Griebel et al. (1999) could not see the same in Wistar-Kyoto rats. An anxiolytic effect of 20 mg/kg of fluoxetine can be seen on the elevated plus-maze after 24 h, but not after 30 min (Griebel et al.,1999). Thus, it seems that different strains, doses, administrations routes and paradigms can change fluoxetine results.

Interestingly, while conducting a pilot study in our laboratory, an anxiolytic effect of fluoxetine, without impairment of locomotor activity, was found in FEP. Later, it was realised that an error had occurred during the preparation of the fluoxetine solution, which ended up being 10 times less concentrated, so that the animals received 0.5 mg/kg, instead of 5 mg/kg, of fluoxetine. In order to confirm this finding, Experiment IV was performed and its results demonstrated that 0.5 mg/kg of fluoxetine indeed decreased levels of trait anxiety and did not change locomotor activity. It has been demonstrated, through *in vivo* brain microdialysis, that low doses of a serotonin reuptake inhibitor, acutely administered, seems to significantly increase levels of extracellular 5-HT in raphe nuclei (Adell and Artigas, 1991; Invernizzi et al. 1992). This increase stimulates presynaptic 5-HT1_A autoreceptors, which are responsible for inhibiting the activity of 5-HT neurons in raphe nuclei. According to the 5-HT hypothesis of anxiety, this inhibition would generate an anxiolytic effect, as acute increases in 5-HT neurotransmission induces anxiety, while a decrease could induce anxiolysis (Griebel et al., 1996).

In summary, our results showed that FEP was able to predict both anxiolytic and anxiogenic effects of clinically used drugs, supporting the validity and appropriateness of this model as a test to investigate the pharmacological activities of different drugs in trait anxiety.

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Figure Legends

Fig. 1. Free-exploratory box with a rat in the novel side (right side). The left side corresponds to the familiar environment.

Fig. 2. Total distance travelled (A) and percentage of time spent in the novel side (B) of the Free-exploratory Paradigm (FEP). DZP: 0.5 mg/kg of diazepam; CTRL: vehicle. FEP1: first exposure to FEP (drug naive rats). FEP2: second exposure to FEP (pharmacologically treated rats). Data are presented as means ± SEM. *$p<0.05$ in relation to FEP1. #$p<0.05$ in relation to CTRL on FEP2.

Fig. 3. Total distance travelled (A) and percentage of time spent in the novel side (B) of the Free-exploratory Paradigm (FEP). PTZ: 20 mg/kg of pentylenetetrazole; CTRL: vehicle. FEP1: first exposure to FEP (drug naive rats). FEP2: second exposure to FEP (pharmacologically treated rats). Data are presented as means ± SEM. *$p<0.05$ in relation to FEP1. #$p<0.05$ in relation to CTRL on FEP2.

Fig. 4. Total distance travelled (A) and percentage of time spent in the novel side (B) of the Free-exploratory Paradigm (FEP). FLX5: 5 mg/kg of fluoxetine; CTRL: vehicle. FEP1: first exposure to FEP (drug naive rats). FEP2: second exposure to FEP (pharmacologically treated rats). Data are presented as means ± SEM. *$p<0.05$ in relation to FEP1. #$p<0.05$ in relation to CTRL on FEP2.

Fig. 5. Total distance travelled (A) and percentage of time spent in the novel side (B) of the Free-exploratory Paradigm (FEP). FLX0.5: 0.5 mg/kg of fluoxetine; CTRL: vehicle. FEP1: first exposure to FEP (drug naive rats). FEP2: second exposure to FEP (pharmacologically treated rats). Data are presented as means ± SEM. *$p<0.05$ in relation to FEP1. #$p<0.05$ in relation to CTRL on FEP2.

Figure 1

Figure 2

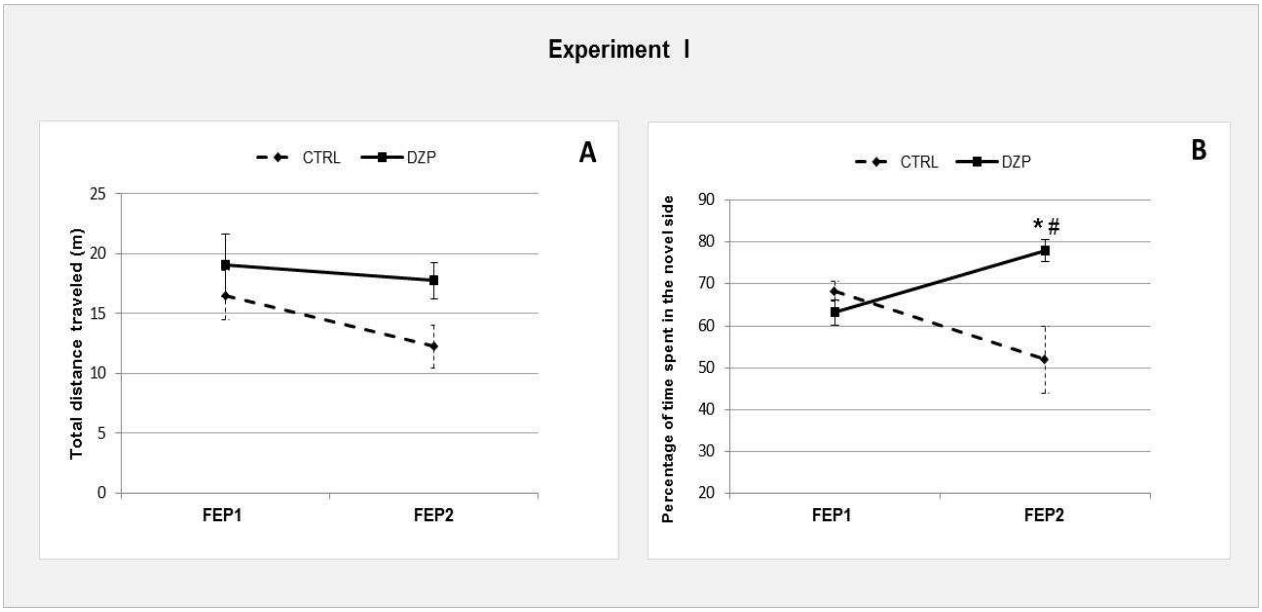

Experiment I
A
CTRL
DZP
Total distance traveled (m)
25
20
15
10
5
0
FEP1
FEP2
B
CTRL
DZP
Percentage of time spent in the novel side
90
80
70
60
50
40
30
20
*#
FEP1
FEP2

Figure 3

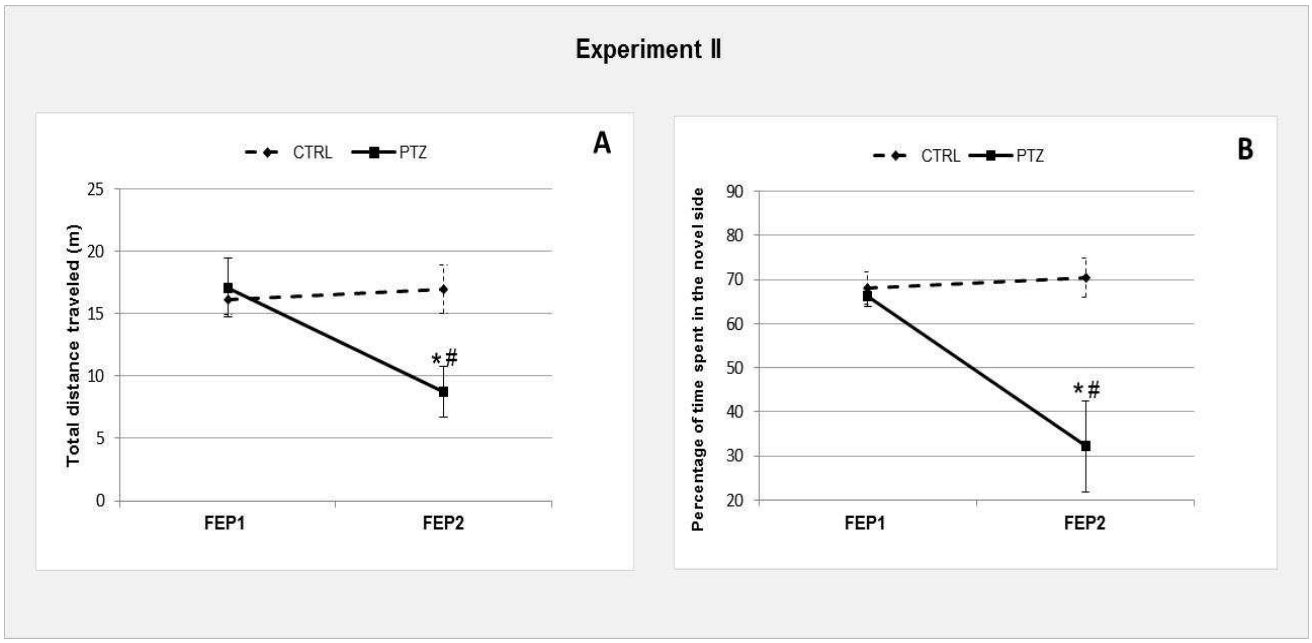

Experiment II
A
CTRL
PTZ
Total distance traveled (m)
25
20
15
10
5
0
*#
FEP1
FEP2
B
CTRL
PTZ
Percentage of time spent in the novel side
90
80
70
60
50
40
30
20
*#
FEP1
FEP2

Figure 4

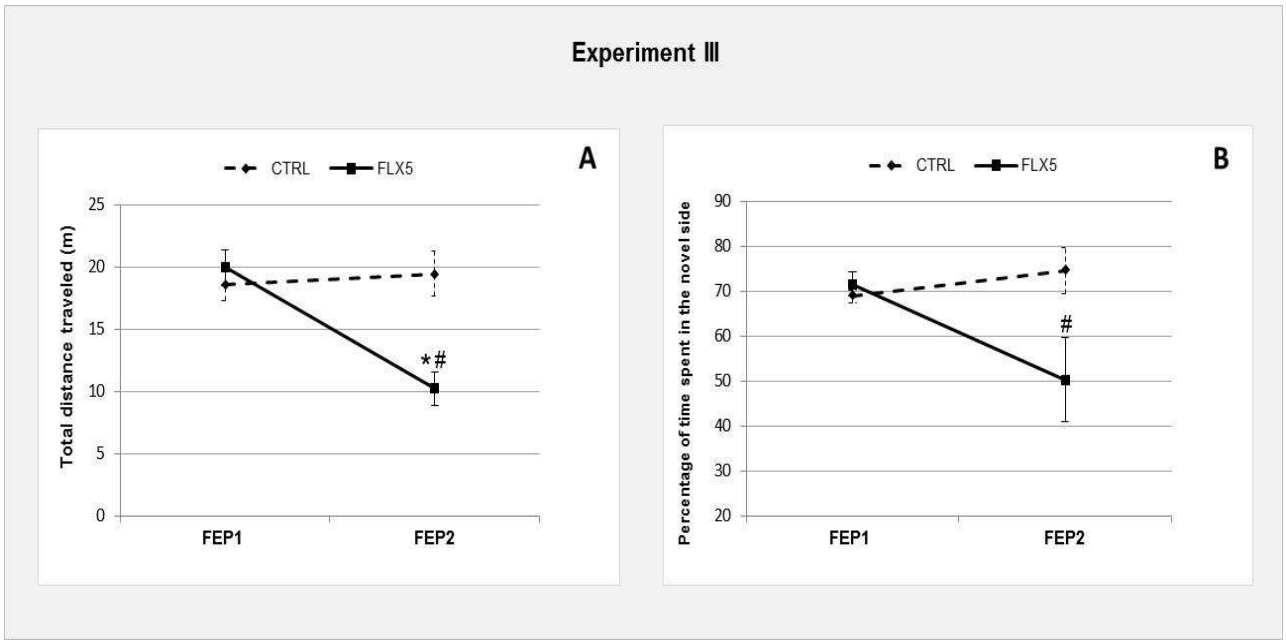
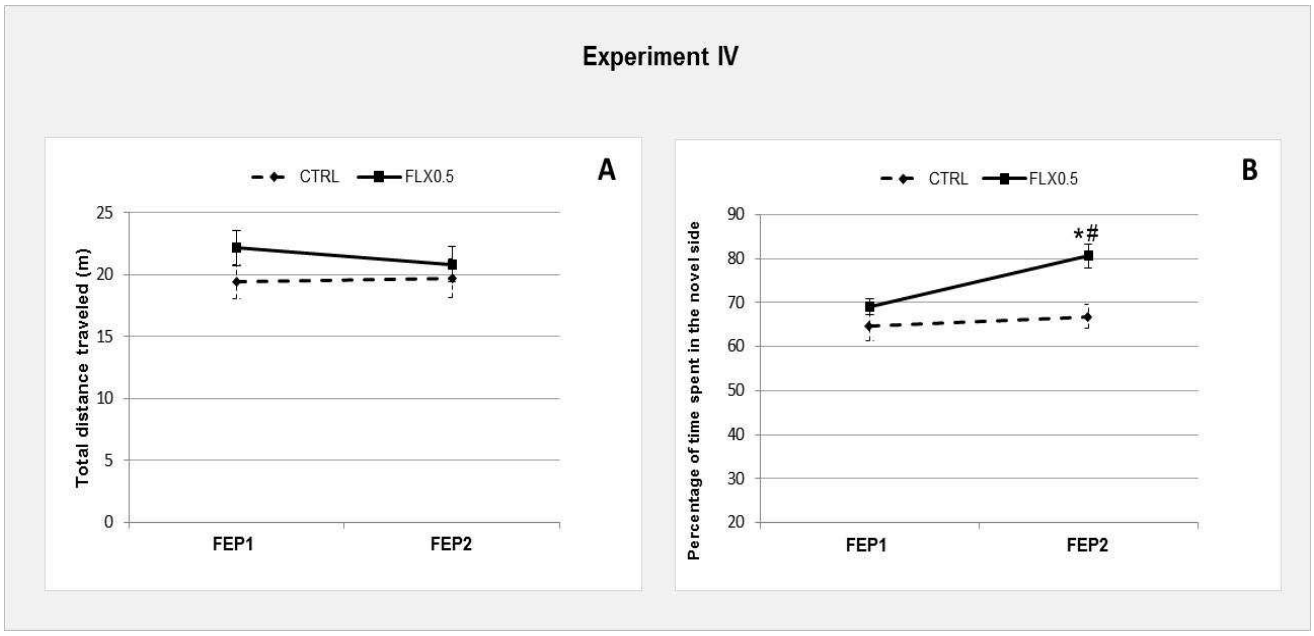

Experiment III
A
CTRL
FLX5
Total distance traveled (m)
25
20
15
10
5
0
*#
FEP1
FEP2
B
CTRL
FLX5
Percentage of time spent in the novel side
90
80
70
60
50
40
30
20
#
FEP1
FEP2

Figure 5


Experiment IV
A
CTRL
FLX0.5
Total distance traveled (m)
25
20
15
10
5
0
FEP1
FEP2
B
CTRL
FLX0.5
Percentage of time spent in the novel side
90
80
70
60
50
40
30
20
*#
FEP1
FEP2